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HIV Controllers Exhibit Enhanced Frequencies of Major Histocompatibility Complex Class II Tetramer$^+$ Gag-Specific CD4$^+$ T Cells in Chronic Clade C HIV-1 Infection

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ABSTRACT Immune control of viral infections is heavily dependent on helper CD4$^+$ T cell function. However, the understanding of the contribution of HIV-specific CD4$^+$ T cell responses to immune protection against HIV-1, particularly in clade C infection, remains incomplete. Recently, major histocompatibility complex (MHC) class II tetramers have emerged as a powerful tool for interrogating antigen-specific CD4$^+$ T cells without relying on effector functions. Here, we defined the MHC class II alleles for immunodominant Gag CD4$^+$ T cell epitopes in clade C virus infection, constructed MHC class II tetramers, and then used these to define the magnitude, function, and relation to the viral load of HIV-specific CD4$^+$ T cell responses in a cohort of untreated HIV clade C-infected persons. We observed significantly higher frequencies of MHC class II tetramer-positive CD4$^+$ T cells in HIV controllers than progressors ($P = 0.0001$), and these expanded Gag-specific CD4$^+$ T cells in HIV controllers showed higher levels of expression of the cytolytic proteins granzymes A and B. Importantly, targeting of the immunodominant Gag41 peptide in the context of HLA class II DRB1*1101 was associated with HIV control ($r = -0.5$, $P = 0.02$). These data identify an association between HIV-specific CD4$^+$ T cell targeting of immunodominant Gag epitopes and immune control, particularly the contribution of a single class II MHC-peptide complex to the immune response against HIV-1 infection. Furthermore, these results highlight the advantage of the use of class II tetramers in evaluating HIV-specific CD4$^+$ T cell responses in natural infections.

IMPORTANCE Increasing evidence suggests that virus-specific CD4$^+$ T cells contribute to the immune-mediated control of clade B HIV-1 infection, yet there remains a relative paucity of data regarding the role of HIV-specific CD4$^+$ T cells in shaping adaptive immune responses in individuals infected with clade C, which is responsible for the majority of HIV infections worldwide. Understanding the contribution of HIV-specific CD4$^+$ T cell responses in clade C infection is particularly important for developing vaccines that would be efficacious in sub-Saharan Africa, where clade C infection is dominant. Here, we employed MHC class II tetramers de-

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signed to immunodominant Gag epitopes and used them to characterize CD4+ T cell responses in HIV-1 clade C infection. Our results demonstrate an association between the frequency of HIV-specific CD4+ T cell responses targeting an immunodominant DRB1*11-Gag41 complex and HIV control, highlighting the important contribution of a single class II MHC-peptide complex to the immune response against HIV-1 infections.

KEYWORDS CD4 T helper cells, MHC class II tetramers, human immunodeficiency virus, HIV

Detailed characterization of immune cells that contribute to the suppression of HIV replication is critical to both vaccine design and therapeutic approaches. Although both CD4+ and CD8+ T cells mediate cellular immune responses to HIV, most research efforts have focused on understanding the role of CD8+ T cells. In contrast, the role of CD4+ T cells is less well defined. CD4+ T cells contribute to the immune control of many viral infections (1–6), implying that they may be similarly important for HIV immunity. Indeed, recent studies in individuals with HIV clade B infection have highlighted the important role of HIV-specific CD4+ T cells as effectors of viral immunity (7, 8), in addition to being orchestrators of CD8+ T cell and B cell responses via helper signals (9).

Although CD4+ T cell responses remain relatively understudied, emerging evidence suggests that they play a more active role in controlling HIV disease progression than was previously thought. A recent study of simian immunodeficiency virus provided the first direct evidence of a CD4+ T cell-driven escape mutation (10). In that study, the investigators identified a unique postbreakthrough mutation in the Gag region targeted only by CD4 responses, which abrogated CD4+ T cell recognition (10). In humans, indirect evidence generated by a computational approach identified HLA class II-associated polymorphisms and linked predicted epitopes within adaptation sites to CD4+ T cell-driven immune escape (11). Furthermore, recent studies conducted in predominantly Caucasian populations with clade B HIV infection showed that targeting of Gag by CD4+ T cell responses restricted by some HLA-DRB1 alleles, such as DRB1*15:02, 13:01 and DQB*:06, was associated with low levels of viremia, whereas the CD4 responses restricted by the DRB1*03:01 allele were linked to high levels of viremia. The plausible explanation for the difference was that DRB1 alleles associated with low viremia had the capacity to present multiple epitopes at low functional avidity compared to alleles associated with high viremia, where a significantly lower number of promiscuous peptide restrictions are observed (12–14). Taken together, these data underscore the impact of HIV-specific CD4+ T cell responses on the control of HIV replication.

In contrast to these studies in individuals with clade B virus infection, much less is known about the role of CD4+ T cell responses in individuals with clade C HIV infection. An improved understanding of the immune responses to clade C is particularly important for the development of vaccines that would be efficacious in sub-Saharan Africa, where subtype C viruses, which account for more than 50% of infections globally, are most dominant (15). A few studies of clade C have identified immunodominant class II-restricted CD4+ T cell epitopes, but the restricting alleles for these responses remain unknown (16, 17). The lack of knowledge about the restricting alleles has made it difficult to synthesize class II tetramers that could be used to conduct more comprehensive CD4+ T cell studies without relying on specific effector functions. Most clade C studies have used gamma interferon (IFN-γ) intracellular cytokine staining (ICS) or enzyme-linked immunosorbent spot (ELISPOT) assays to study CD4+ T cell responses (17). This approach is limited in scope because it can detect the responses only of IFN-γ-secreting CD4+ T cells and is therefore more likely to miss other CD4+ T cell subsets that are defined by different signature cytokines, such as Th-2, Th-17, and T follicular helper cells and regulatory T cells.

To investigate if virus-specific CD4+ T cells play an active antiviral role in the pathogenesis of clade C HIV infection, we first screened the entire HIV-1 clade C proteome for immunodominant HIV-specific CD4+ T cell epitopes and performed class II restriction studies to identify the most frequent class II DRB1 alleles in an initially untreated study population predominantly of Zulu/Xhosa ethnicity in a region where clade C is endemic. The screening data were used to synthesize major histocompatibility complex (MHC) class II tetramers to the most immunodominant CD4+ T cell epitopes. The tetramers were then used to thoroughly examine the frequency and function of HIV-specific CD4+ T cell responses during chronic untreated HIV-1 clade C infection. Our data demonstrate that the frequency of immunodominant Gag-specific CD4+ T cells measured by the tetramers is inversely associated with the contemporaneous viral load. These data highlight the important contribution of HLA class II-restricted epitope-specific CD4+ T cells to the immune response against HIV-1 clade C infection and indicate that MHC class II tetramers are a sensitive tool for interrogating HIV-specific CD4+ T cell responses in natural infections.

RESULTS

To better understand the role of HIV-specific CD4+ T cell responses in HIV control and disease progression, we comprehensively investigated the specificity and functional properties of these responses in the setting of chronic clade C HIV-1 infection using IFN-γ ELISPOT assays and multiparameter flow cytometry with MHC class II tetramers. Modified IFN-γ ELISPOT assays were used to identify immunodominant HIV-specific CD4+ T cell epitopes and restricting alleles in our cohort with chronic infection to inform the production of appropriate MHC class II tetramers. MHC class II tetramers were then utilized to detect low-frequency populations of antigen-specific CD4+ T cells (which may be missed in conventional peptide stimulus-based assays). This approach enabled the direct *ex vivo* characterization of antigen-specific HIV-specific CD4+ T cell responses targeting immunodominant Gag epitopes.

Immunodominance hierarchy of CD4+ T cell responses in chronic clade C infection. Here, we evaluated a cohort of 72 untreated individuals chronically infected with HIV clade C. HIV-specific CD4+ T cell responses against a panel of 410 pooled peptides spanning the entire HIV-1 clade C consensus sequence were initially screened using the IFN-γ ELISPOT megamatrix assay. Results from the initial megamatrix assay screening were validated using confirmatory IFN-γ ELISPOT assays at the single-peptide level. Our data demonstrate that HIV-specific CD4+ T cell responses in chronic clade C infection dominantly target the Gag protein (Fig. 1A). The most commonly targeted region in Gag was the p24 subprotein (20/63 peptides), while the p17 and p15 regions of Gag were subdominantly targeted by CD4+ T cells (12/63 peptides each). The p24 region of Gag has also been shown to be immunodominant for HIV-specific CD8+ T cell responses, and these responses have previously been associated with viral control (18). However, no correlation between the breadth of Gag-specific CD4+ T cell responses (Spearman $r = -0.17$, $P = 0.42$) as well as the magnitude of these responses (Spearman $r = 0.22$, $P = 0.30$), as measured by ELISPOT assays, and the contemporaneous viral load was observed. At the epitope level, our data showed that Gag peptide 41 (Gag41) within the p24 subunit is the most immunodominant peptide, with over 40% of the subjects in our cohort showing a detectable response to this peptide (Table 1). A previous study found Gag6 in p17 to be the most dominant epitope (17). The difference may be due to the different proportions of controllers and progressors between the two studies.

The second most commonly targeted epitope was Gag40 (23%). Interestingly, only two responders targeted both the adjacent peptides, indicating the presence of distinct epitopes rather than targeting of the shared overlap represented in each peptide. Gag6, Gag25, and Gag37 were targeted independently by 20% of the 30 responders (Table 1). Additional immunodominant epitopes were located within the Nef region of the proteome (7/63 peptides). The results highlight a large number of detectable HIV-specific CD4+ T cell responses across the HIV proteome dominantly targeting Gag and

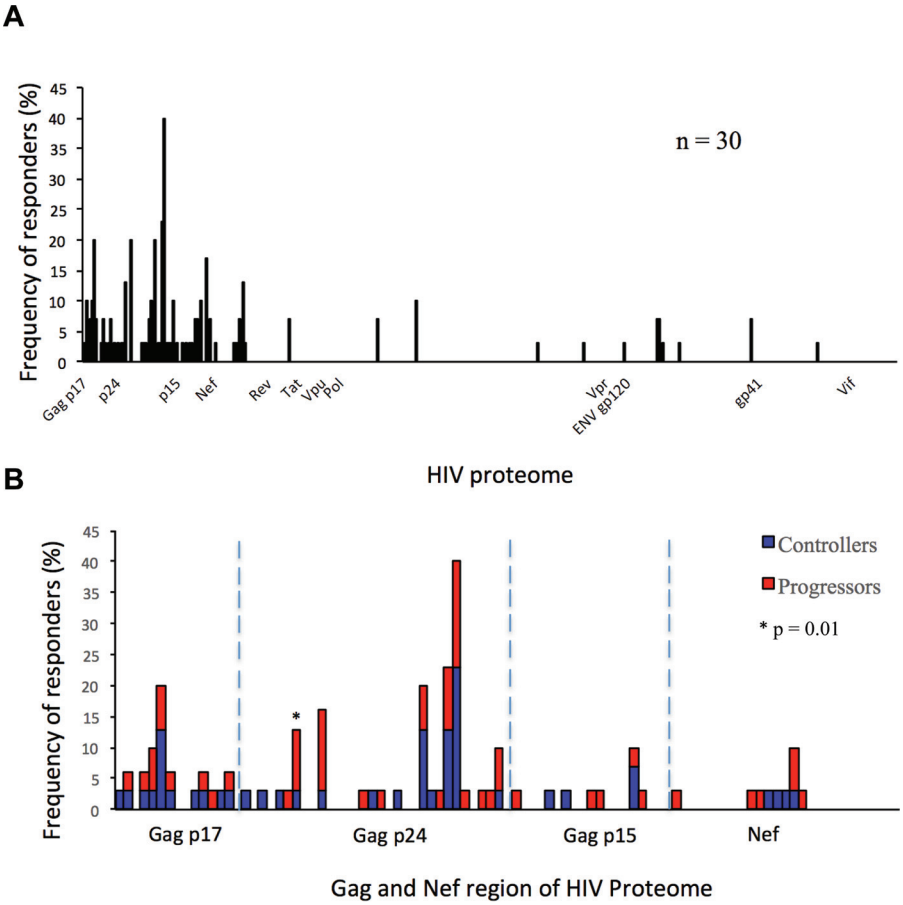

FIG 1 (A) Frequency of targeting of HIV-specific CD4$^+$ T cell responses to overlapping peptides across the HIV-1 proteome. HIV-specific CD4$^+$ T cell responses against a panel of 410 OLPs spanning the entire HIV proteome were screened. The labels on the *x* axis indicate the start of the relevant HIV protein or subprotein. The percentages of responders (30/72 individuals screened) with epitope-specific CD4$^+$ T cell responses are shown. (B) Percentages of epitope-specific CD4$^+$ T cell responses targeting the respective OLPs across the Gag and Nef proteins between controllers ($n = 13$) and progressors ($n = 17$) from a chronically infected cohort. No significant differences were observed between the two groups ($P = 0.65$, based on a nonparametric two-tailed *t* test). Further analysis of each individual response indicated a significant (*, $P = 0.01$, Fisher's exact test) more predominant targeting of Gag25 in the Gag p24 region by progressors than by controllers.

Nef, findings consistent with those of previous studies (17). We observed that Gag-specific CD4$^+$ T cell responses are immunodominant and are directed at multiple distinct epitopes, with very few detectable CD4$^+$ T cell responses to Env or accessory proteins being detected. This is in contrast to the findings of studies in individuals with chronic clade B infection, which have detected CD4$^+$ T cell responses targeting multiple epitopes within Env gp120 and rarely targeting overlapping peptide (OLP) 25 in Gag (19), suggesting that the immunodominance hierarchy of HIV-specific CD4$^+$ T cell targeting may be influenced by the infecting clade (20). Moreover, our study has identified 22 peptides that have not been described to be immunodominant epitopes in previous studies of HIV-1 clade C.

As the cohort was stratified into controllers and progressors, we next evaluated whether HIV-specific CD4$^+$ T cell responses within these two groups may be directed at different epitopes. However, there was no significant difference between the two groups ($P = 0.65$) (Fig. 1B). A few epitopes were exclusively targeted by each group at low frequencies of 3%. There were particular epitopes that, albeit they were targeted by both groups, were more targeted by progressors, such as Gag25, which was targeted by only 3% of controllers and 13% of progressors (Fischer's exact test, $P = 0.01$). Notably, the highly immunodominant Gag41 epitope was targeted by both controllers

TABLE 1 Immunodominant peptides targeted by study participants[a]

Peptide	Protein	Location in HXB2 (amino acids)	OLP sequence	% of responders targeting OLP[b]
41	p24	164–181	YVDRFFKTLRAEQATQDV	40
40	p24	156–173	GPKEPFRDYVDRFFKTLR	23
6	p17	37–51	ASRELERFALNPGLL	20
25	p24	46–62	GATPQDLNTMLNTVGGH	20
37	p24	133–150	WIILGLNKIVRMYSPVSI	20
62	p15	85–102	FLQSRPEPTAPPAESFRF	17
22	p24	23–40	WVKVIEEKAFSPEVIPMF	13
81	Nef	104–121	KKRQEILDLWVYHTQGYF	13
2	p17	9–26	RGGKLDKWEKIRLRPGGK	10
5	p17	32–46	KHLVWASRELERFAL	10
35	p24	117–134	WMTSNPPVPVGDIYKRWI	10
46	p24	200–217	TILRALGPGASLEEMMTA	10
60	p15	72–89	GKIWPSHKGRPGNFLQSR	10
168	RT	15–32	GMDGPKVKQWPLTEEKIK	10
4	p17	25–41	GKKHYMLKHLVWASREL	7
7	p17	42–58	ERFALNPGLLETSEGCK	7
11	p17	70–86	TGTEELRSLYNTVATLY	7
14	p17	92–110	IEVRDTKEALDKIEEEQNK	7
34	p24	109–126	STLQEQIAWMTSNPPVPV	7
57	p15	51–68	WKCGKEGHQMKDCTERQA	7
58	p15	59–75	QMKDCTERQANFLGKIW	7
64	p15	103–123	RFEETTPAPKQEPKDREPL	7
79	Nef	88–105	SFFLKEKGGLEGLIYSKK	7
104	Rev	72–88	PLQLPPIERLHIDCSES	7
148	Protease	22–40	RANSPTSRELQVRGDNPR	7
289	gp120	1–7	MRVMGIQRNCQQWWRW	7
290	gp120	7–27	QRNCQQWWRWGILGFWML	7
336	gp120	351–371	EHFPNKTIKFAPSSGGDLEI	7

[a]Immunodominance hierarchy of frequently targeted HIV-specific CD4+ T cell responses in the cohort with chronic clade C HIV infection.

[b]Percentages were calculated according to the results for those individuals that demonstrated an HIV-specific CD4+ T cell response.

and progressors (23% and 17% responders, respectively), thus providing sufficient coverage of both groups for in-depth quantitative and qualitative evaluation of the Gag41-specific CD4+ T cell responses by MHC class II tetramers and the relationship of these responses to the viral load.

HLA-DRB1*13:01 and HLA-DRB1*11:01 are the most frequent alleles among CD4+ T cell responders. Having identified immunodominant epitopes targeted in chronic clade C infection, we next performed HLA class II typing as described in the Materials and Methods to determine the HLA class II allele frequencies among the subjects with detectable responses by the IFN-γ ELISPOT assay ($n = 30$) (Fig. 2A). Our data suggest that individuals with the strongest HIV-specific CD4+ T cell responses possessed the class II HLA-DRB1*13:01 allele ($n = 12$, 21% of responders). The next most common alleles were DRB1*11:01 and DRB1*03:02, expressed by 14% and 9% of responders, respectively. Interestingly, the most common DRB1 alleles among the responders in our study were also the most frequent in the larger cohort with chronic clade C infection ($n = 439$), whereby DRB1*03:02 was the most common allele observed ($n = 146$, 33%), followed by DRB1*11:01 ($n = 111$, 25%) and DRB1*13:01 ($n = 102$, 23%) (16). It is important to note that DRB1*13 alleles have been reported to confer a protective effect (13), with DRB1*13:03 in particular having been shown to mediate protection independently of ethnicity, sex, and viral clade (16).

HLA-DRB1 allele restriction characteristics of HIV-specific CD4+ T cell responses in clade C infection. Having defined the frequencies of these class II alleles in this cohort with chronic clade C infection, we next identified DRB1 restricting alleles using the HLA restriction assay previously described by Ranasinghe et al. (12). We observed high levels of peptide promiscuity, which is known to be a distinctive feature of antigen-specific CD4+ T cell recognition (21), with the presentation of a single

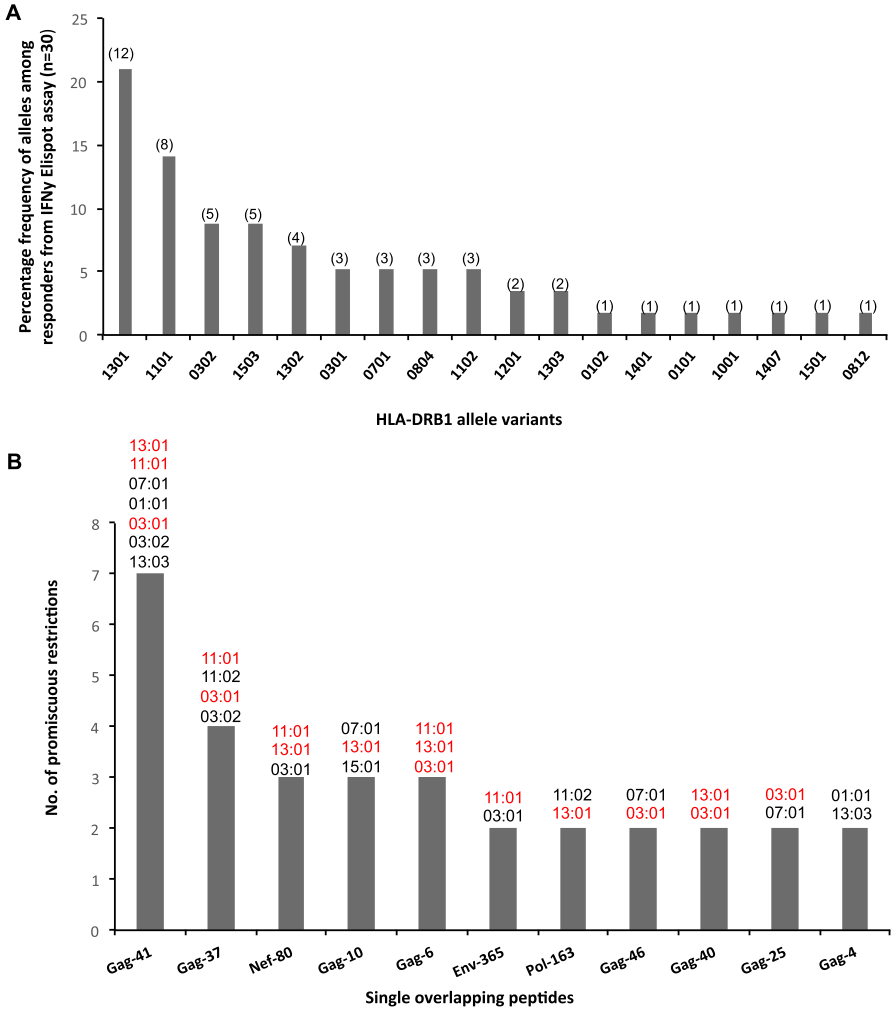

FIG 2 HLA class II restriction characteristics of HIV-specific CD4$^+$ T cell responses in a cohort with chronic clade C infection. (A) Frequency of various HLA-DRB1 allele variants in CD4$^+$ T cell responders (n = 30). The number of responders possessing each allele is indicated in parentheses above each bar. (B) HLA-DRB1 allele restriction characteristics of HIV-specific CD4$^+$ T cell responses in individuals with clade C infection (promiscuous epitopes). The restricting HLA alleles for each overlapping peptide are indicated above each bar. Alleles that are highlighted in red were used to generate MHC class II tetramers.

peptide on numerous HLA class II variants. Gag41 in the Gag p24 region, previously identified to be the epitope most frequently targeted by HIV-specific CD4$^+$ T cells in clade B (17, 19) and also shown to be the most frequently targeted epitope in this cohort, exhibited the highest levels of promiscuity, being restricted by 7 different HLA-DRB1 variants (Fig. 2B). The promiscuity in peptide binding is likely mediated by the open conformation of HLA class II, which allows long peptides recognized by CD4$^+$ T cells to extend beyond the HLA binding groove (12). Despite the high degree of HLA-DRB1 binding promiscuity, marked differences in the number of peptides that were restricted by each DRB1 variant were observed. Variants such as DRB1*14:01 and DRB1*04:01 had no detectable contribution to overall peptide restrictions, whereas DRB1*13:01 and DRB1*11:01 restricted 9 and 4 HIV-specific peptides, respectively.

Use of HLA class II tetramers for sensitive detection of HIV-specific CD4$^+$ T cells. Having identified immunodominant CD4$^+$ T cell epitopes and the most common restricting HLA DRB1 allele variants in a large number of clade C responders (30/72 individuals tested), the information was used to successfully generate and validate the following tetramers: DRB1*03:01 (Gag37), DRB1*11:01 (Gag41), and DRB1*13:01

TABLE 2 MHC class II tetramers[a] used for analysis of tetramer+ CD4+ T cells

HIV tetramer	Sequence	OLP	Clade
HLA-DRB1*03:01	WIILGLNKIVRMYSPVI	Gag37	C
HLA-DRB1*11:01	YVDRFFKTLRAEQATQDV	Gag41	C
HLA-DRB1*13:01	WIILGLNKIVRMYSPVI	Gag37	C
HLA-DRB1*13:01	YVDRFFKTLRAEQATQDV	Gag41	C

[a]MHC class II tetramers were produced according to allele variants associated with the most frequently targeted peptides and the highest number of responders.

(Gag37 and Gag41). Each of these was conjugated to both phycoerythrin (PE) and allophycocyanin (APC) (Table 2). The newly synthesized tetramers were then used to evaluate the role of HIV-specific CD4+ T cell responses in the pathogenesis of clade C HIV-1 infection. To our knowledge, these particular MHC class II tetramers have not been synthesized before in the setting of clade C infection. Furthermore, our study is the first to generate and utilize the DRB1*11:01 tetramer to Gag41 to study CD4+ T cell responses.

Given the low frequency of HIV-specific CD4+ T cell responses (22), a dual PE and APC tetramer-staining strategy with gating on double-staining cells was used to increase our ability to detect genuine tetramer-positive (tetramer+) cells and minimize nonspecific background staining (Fig. 3A). Class II tetramer staining on CD8+ T cells was also assessed for each sample to ensure that there was minimal nonspecific tetramer binding (Fig. 3A). The specificity was confirmed by staining 3 HIV-negative samples,

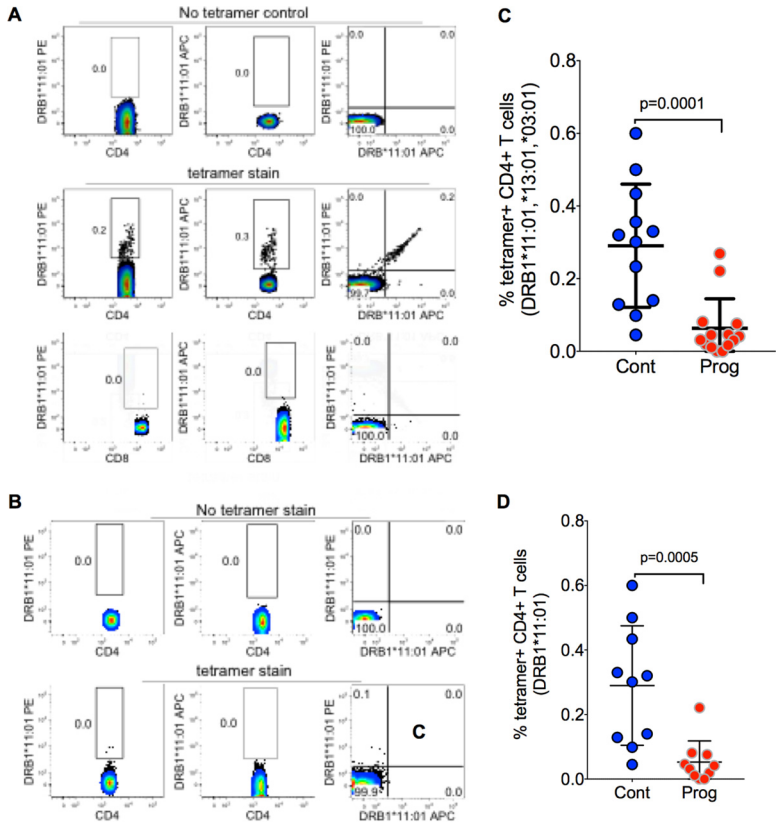

FIG 3 Frequency of tetramer-positive CD4+ T cells in chronically infected controllers and progressors and HIV-negative subjects. (A, B) Representative flow plots indicating a dual PE and APC tetramer-staining strategy with gating on double-stained cells, which was used to increase the ability to detect genuine tetramer+ cells and minimize nonspecific background staining on CD8+ T cells, from an HIV-infected individual (A) and from an HIV-negative individual (B). (C, D) Differences in the percentage of CD4+ tetramer+ T cells between controllers (Cont) and progressors (Prog) for all class II DRB1*03:01, DRB1*11:01, and DRB1*13:01 tetramers utilized ($P = 0.0001$) (C) and for only the DRB1*11:01 tetramer ($P = 0.0005$) (D).

none of which stained positive (representative data are shown in Fig. 3B). Low-frequency class II DRB1*03:01, DRB1*11:01, and DRB1*13:01 tetramer binding CD4$^+$ T cells were readily detectable in 11 controllers expressing the requisite DRB1 alleles at a median frequency of 0.31% (interquartile range [IQR], 0.045% to 0.41%) of the total CD4$^+$ T cells. Notably, the median frequency of class II tetramer binding cells in 10 chronic progressors expressing the requisite DRB1 was 0.036% (IQR, 0.015% to 0.27%; $P = 0.0001$), which was 10-fold lower than that in controllers (Fig. 3C). Analysis of the DRB1*11-Gag41 tetramer binding responses also showed a higher frequency in controllers than progressors ($P = 0.0005$) (Fig. 3D). The controllers and progressors were matched for expression of DRB1*11, with an equal number of each expressing this allele. These data suggest that HIV-specific CD4$^+$ T cell responses targeting immunodominant Gag41 in the context of DRB1*11 expression are associated with the maintenance of low levels of viremia during chronic clade C HIV infection.

Dominance of monospecific HIV-specific CD4$^+$ T cell responses in controllers and progressors. Given that tetramers can bind antigen-specific cells irrespective of function, we next examined the range of CD4$^+$ T cell functionalities that constitute tetramer$^+$ responses. Peripheral blood mononuclear cells (PBMCs) were stimulated with an HIV clade C Gag pool or the Gag41 or Gag37 OLP. Multicolor flow cytometry was used to detect and enumerate key effector CD4$^+$ T cell functions in individuals with tetramer$^+$ responses. We measured markers that define key CD4$^+$ T cell functional subsets. Our analysis included the degranulation marker CD107a, which was used to enumerate cytolytic CD4$^+$ T cells (7); IFN-γ and interleukin-2 (IL-2), which were used to identify Th-1 helper cells (23); and IL-21, which was included to identify cells that augment B cell and CD8$^+$ T cell effector functions (9, 24). To investigate if any of the measured functions impact virus suppression, we compared the expression level of each marker in controllers and progressors. The HIV controllers exhibited higher mean/median levels of expression of each of the four functions than the progressors in response to stimulation with a mix of Gag peptides (Fig. 4A and B), in response to the immunodominant Gag41 (Fig. 4C), or in response to staphylococcal enterotoxin B (SEB) (Fig. 4D), yet only the response to SEB reached statistical significance after correction for multiple comparisons. Previous studies have shown that the ability to simultaneously elaborate multiple functions is associated with protective immunity against several viral infections (reviewed by Harari et al. [25]); moreover, multifunctional CD4$^+$ T cell responses were associated with protection in the RV144 trial (26–28). Therefore, we next examined if certain combinations of functions were associated with slow HIV disease progression. Combinatorial polyfunctionality analysis showed a preponderance of monospecific responses for both controllers and progressors (Fig. 4E and F). The high frequency of monofunctional cells highlights the limitation of assays which rely on a single function as a surrogate for the entire HIV-specific CD4$^+$ T cell response.

Gag-specific tetramer$^+$ CD4$^+$ T cell responses inversely correlated with the HIV viral load. Given the observed link between CD4$^+$ T cell responses and the maintenance of low levels of viremia, we next interrogated whether maintenance of Gag-specific (tetramer$^+$) CD4$^+$ T cell responses during chronic HIV infection influences disease progression. The frequency of Gag-specific CD4$^+$ T cells measured by four MHC class II tetramers negatively correlated with the contemporaneous viral load (Spearman $r = -0.5$, $P = 0.005$) when the data for both controllers and progressors were analyzed together (Fig. 5A). However, in separate analyses of the responses of controllers (Spearman $r = 0.3$, $P = 0.3$) and progressors (Spearman $r = -0.01$, $P = 0.9$), the correlation did not reach statistical significance, which may have been due to the small sample size. Furthermore, a negative correlation between the frequency of tetramer$^+$ CD4$^+$ T cells targeting DRB1*11-Gag41 and the contemporaneous viral load (Spearman $r = -0.5$, $P = 0.02$) was also observed (Fig. 5B). Interestingly, the levels of IFN-γ secretion measured by ICS exhibited a similar negative correlation with the viral load (Spearman $r = -0.7$, $P = 0.003$) (Fig. 5C). However, the levels of IL-2, IL-21, and CD107a

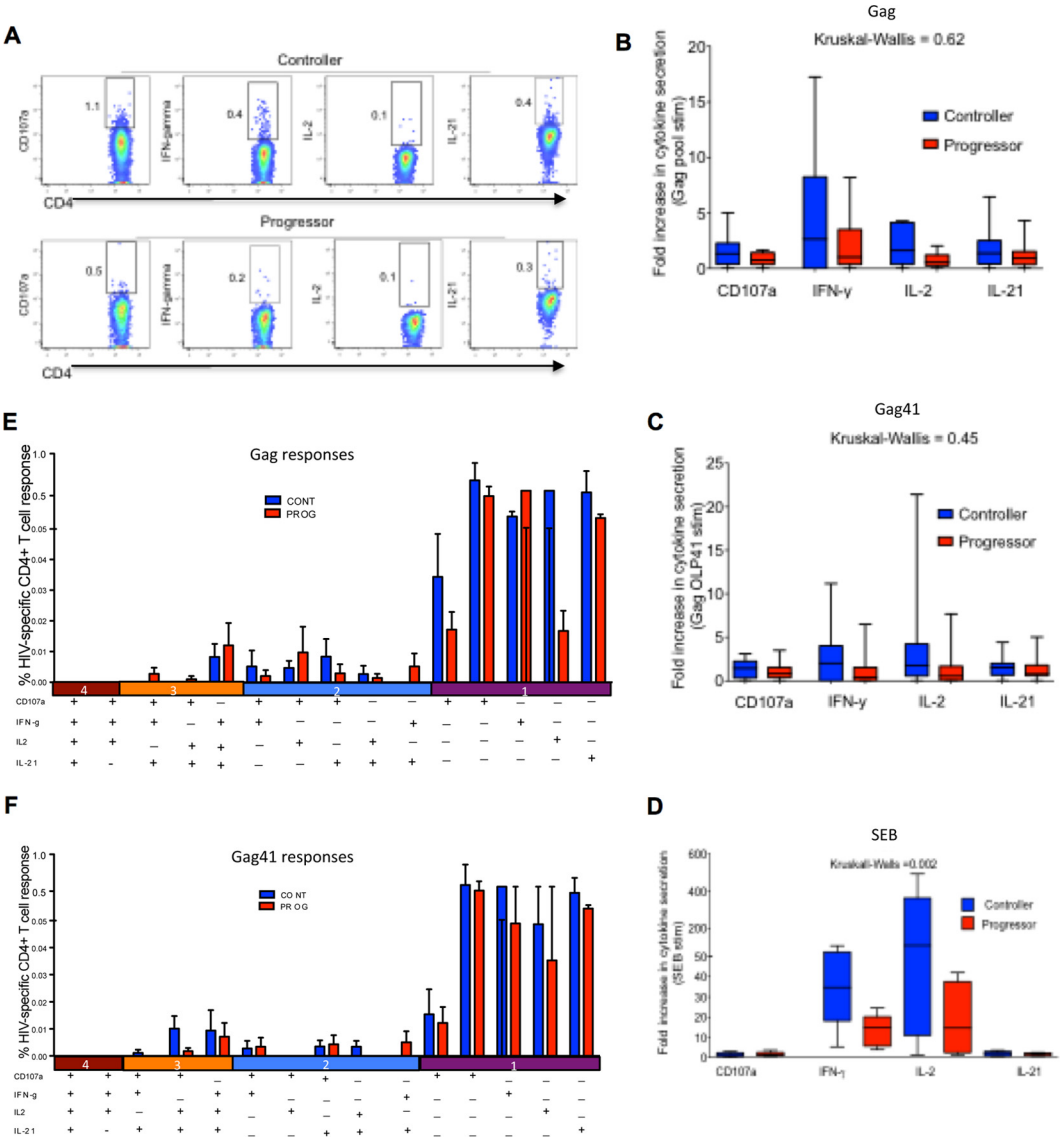

FIG 4 HIV-specific CD4+ T cell polyfunctional responses. (A) Graphical representation of intracellular cytokine staining of controller and progressor individuals on the basis of CD107a stimulation. (B to D) The expression of CD107a, IFN-γ, IL-2, and IL-21 in 14 chronically infected subjects divided into controllers and progressors in response to Gag pool stimulation (stim) (B), to Gag41 stimulation (C), and to SEB stimulation (D) was measured. (E, F) The bars depict the frequency of CD4+ T cells expressing a combination of the functions indicated below the *x* axis.

secretion did not correlate with the contemporaneous viral load (data not shown). This observation prompted us to examine the relationship between tetramer+ CD4+ T cells and cytokine-positive (cytokine+) cells following stimulation with Gag peptides. Interestingly, only IFN-γ-secreting cells correlated with tetramer+ cells (Spearman $r = 0.7$, $P = 0.001$) (Fig. 5D). There was no correlation between the level of IL-2 or IL-21 secretion and the frequency of tetramer+ responses (Fig. 5E and F), and the correlation between the level of secretion of the degranulation marker CD107a and the frequency of tetramer+ responses was only of borderline significance ($P = 0.04$) (Fig. 5G). The plausible explanation for the lack of a positive correlation between tetramer+ cells and these cytokine+ cells may be that some IL-2- and IL-21-secreting cells recognized different epitopes within Gag or, alternatively, were restricted by other class II alleles.

Overall, these data highlight the heterogeneity of the effector functions of HIV-specific CD4+ T cells and illustrate the advantage of using tetramers to identify

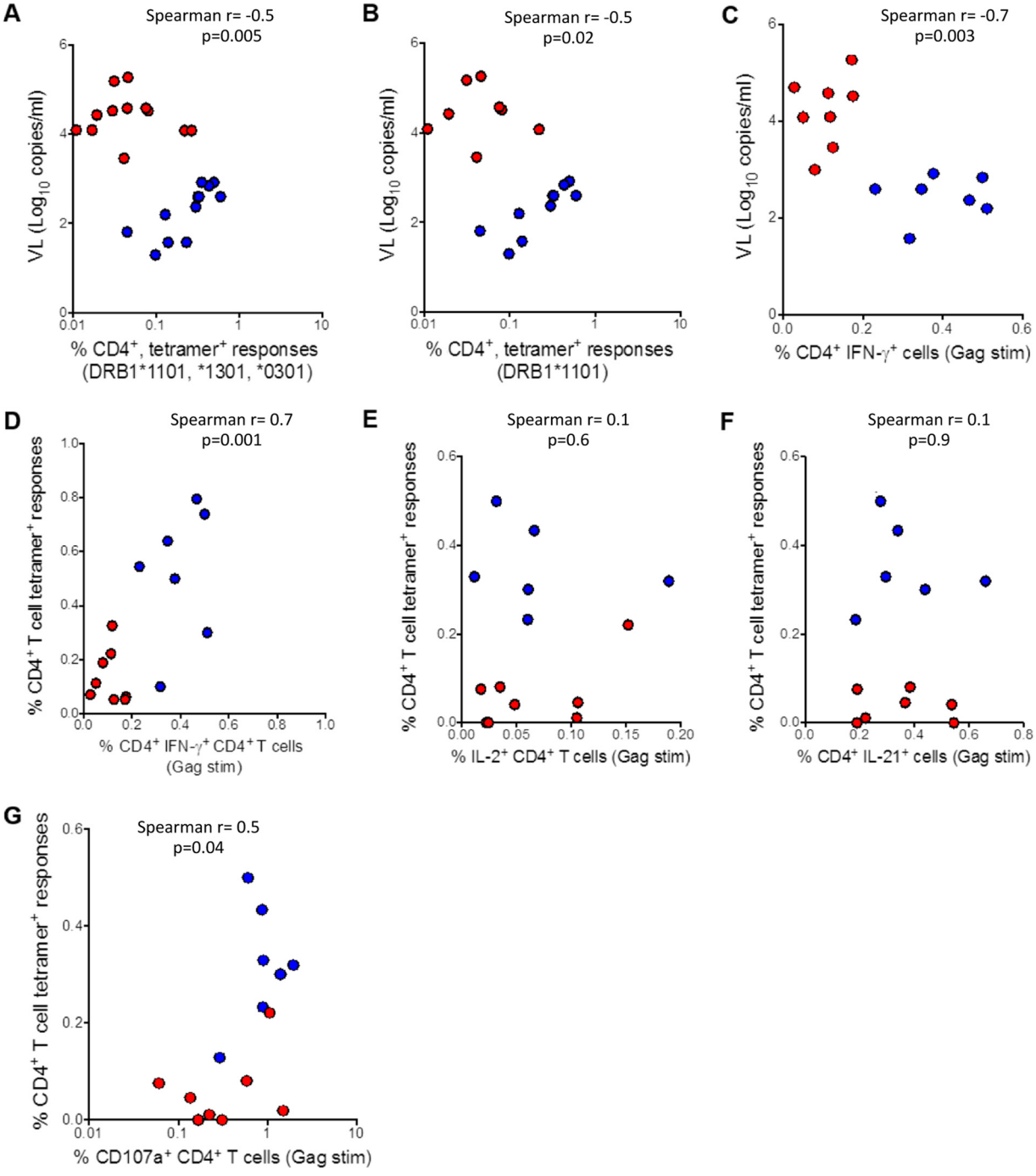

FIG 5 Gag-specific tetramer$^+$ CD4$^+$ T cell responses correlate with markers of HIV disease progression. (A) The frequency of HIV-specific CD4$^+$ T cells measured by four class II tetramers negatively correlated with the contemporaneous viral load (VL) (Spearman $r = -0.5$, $P = 0.005$). (B) A negative correlation between the most dominant Gag41 response (DRB1*11:01) and the contemporaneous viral load was also observed. (C) IFN-γ secretion, measured by ICS, exhibited a similar negative correlation with the viral load (Spearman $r = -0.7$, $P = 0.003$). (D to G) In addition, the relationship between tetramer$^+$ CD4$^+$ T cells and cytokine$^+$ cells following stimulation with HIV peptides was analyzed.

antigen-specific cells without relying on function. Furthermore, these data suggest that immunodominant Gag-specific CD4$^+$ T cell responses are linked with better control of HIV, indicating that these responses may play a role in suppressing HIV replication by antiviral effector functions.

***Ex vivo*-expanded HIV-specific CD4$^+$ T cells from controllers display increased cytolytic activity.** The low-frequency HIV-specific CD4$^+$ T cells detected by class II tetramers may mostly be cells with low-frequency memory responses, which have a low

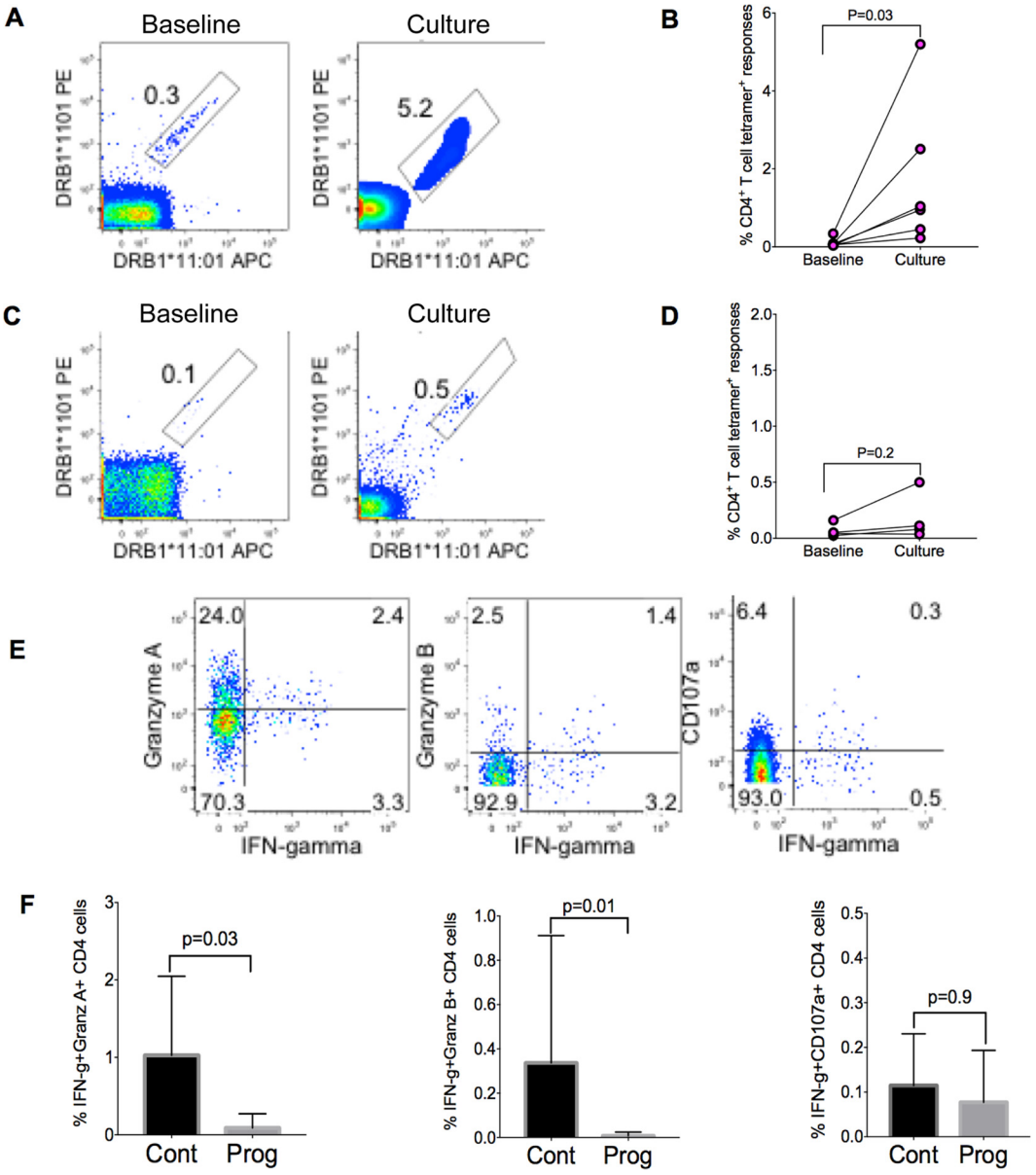

FIG 6 Frequency of tetramer-positive CD4+ T cells in chronically infected controller and progressor individuals following cell expansion. (A, C) Representative flow plots indicating a dual PE and APC tetramer-staining strategy with gating on double-stained cells at the baseline and 2 weeks following expansion of peptide-specific T cell lines from an HIV controller (A) and from an HIV progressor (C). (B, D) Differences in the percentage of CD4+ tetramer+ T cells in 3 controllers, indicating the responses at the baseline and 2 weeks after expansion in culture (red) for all class II DRB1*03:01, DRB1*11:01, and DRB1*13:01 tetramers utilized ($P = 0.02$) (B), and for 3 progressors, using the same principle (D). (E) Representative flow plots indicating the functional responses by IFN-γ-specific CD4+ T cells to granzyme A, granzyme B, and CD107a following 2 weeks of expansion. (F) Comparison of IFN-γ-specific CD4+ T cells to granzyme A ($P = 0.03$), granzyme B ($P = 0.01$), and CD107a ($P = 0.9$) between controllers and progressors.

potential to secrete cytokines *ex vivo*. Moreover, several studies of HIV-specific CD8+ T cell responses have demonstrated that *ex vivo* expansion is needed to accurately measure the cytolytic potential of memory responses (29, 30). We reasoned that this might also be true for HIV-specific CD4+ T cell responses. Thus, we next performed a stimulated expansion of the low-frequency HIV-specific CD4+ T cells that were detected by tetramers and evaluated the cytolytic potential of these expanded cells. Cultured stimulation of HIV-specific CD4+ T cells from controllers resulted in the significant expansion of the tetramer+ populations compared to the levels at the baseline ($P = 0.03$) (Fig. 6A and B), whereas class II tetramer+ populations from progressors expanded

poorly ($P = 0.2$) (Fig. 6C and D). Assessment of the cytolytic activity of *ex vivo*-expanded HIV-specific CD4$^+$ T cells by intracellular cytokine staining (Fig. 6E) showed that controllers had a significantly higher proportion of HIV-specific cells expressing granzyme A ($P = 0.03$) and granzyme B ($P = 0.01$) than progressors (Fig. 6F). No significant differences for CD107a were observed between the groups. Taken together, these data suggest that HIV-specific CD4$^+$ T cells from controllers have higher proliferative and cytolytic functions than those from progressors, suggesting their potential contribution to direct antiviral activity during chronic HIV infection.

DISCUSSION

Together, the data presented here demonstrate an association between the frequency of HIV-specific CD4$^+$ T cell responses targeting distinct immunodominant epitopes in Gag and immune control of clade C HIV viremia. In particular, we show that CD4$^+$ T cells targeting Gag41 in the context of HLA-DRB1*11 are associated with immune control of HIV (in HLA-matched individuals). To our knowledge, this is the first study to demonstrate that CD4$^+$ T cells directed against a single peptide-HLA class II specificity are associated with low levels of HIV viremia. Indeed, although CD8$^+$ T cell targeting of HLA class I-peptide complexes, such as B*27-KK10, is well-known to be associated with the spontaneous control of HIV infection (31, 32), previous studies assessing the contribution of HIV-specific CD4$^+$ T cells to HIV immune control have either focused on the expression of distinct HLA-DRB1 alleles (12–14, 16) or identified particular CD4$^+$ T cell epitopes, such as Gag41, targeted at different stages of disease (17, 19, 20, 33). The finding that there is an association between the frequency of HIV-specific CD4$^+$ T cells targeting the DRB1*11-Gag41 complex and low levels of HIV viremia (in our assessment of 20 HIV-positive HLA-matched individuals) is consistent with our hypothesis that the maintenance of robust HIV-specific CD4$^+$ T cell responses to the Gag p24 region may contribute to the immune control of chronic HIV replication rather than to the fueling of disease progression through infection of activated HIV-specific CD4$^+$ targets.

It is important to note that although the frequency of HIV-specific CD4$^+$ T cells measured by four MHC class II tetramers negatively correlated with the contemporaneous viral load when the data for controllers and progressor were analyzed together, the correlation did not reach statistical significance when the data for each group were analyzed separately. This may have been due to the smaller sample size when the data for each group were analyzed separately. Nevertheless, the fact that controllers maintained higher frequencies of class II tetramer$^+$ CD4 T cells than progressors suggests an association between higher frequencies of virus-specific CD4$^+$ T cell responses and the maintenance of lower viral loads.

Although our analysis of peptide-stimulated HIV-specific CD4$^+$ T cells found that they predominantly secreted IFN-γ directly *ex vivo*, we speculate that *in vivo* these epitope-specific CD4$^+$ T cells may provide superior helper activity to HIV-specific CD8$^+$ T cells that enhances the suppression of HIV replication through a cocktail of cytokine signals or they may directly kill HIV-infected CD4$^+$ T cell and macrophage targets. Indeed, the finding that our *ex vivo*-expanded CD4$^+$ T cell lines expressed granzymes A and B, the expression of which was significantly enhanced in HIV controllers, supports the hypothesis that CD4$^+$ T cells targeting immunodominant Gag peptides may exhibit functions that contribute to the immune control of HIV (7, 34). However, since it is not possible to determine cause versus consequence in our cross-sectional analysis, it remains unclear whether weak HIV-specific T cell responses are a result of ongoing viral replication or, alternatively, whether the superior frequency and superior function of these responses contribute to effective immune control of the virus or some T cell specificities may simply be better preserved in individuals with low levels of viremia and low levels of immune activation.

The improved elucidation of HIV-specific CD4$^+$ T cell responses and an enhanced understanding of how such CD4$^+$ T cells may promote the generation of protective CD8$^+$ T cell and B cell responses or mediate a direct antiviral role are highly relevant

TABLE 3 Summary of clinical characteristics of study participants[a]

Stratum definition (viral load)	No. of subjects	Median (range) viral load (copies/ml)	Median (range) CD4 count (cells/μl)	No. of subjects tested by:		
				Screening (ELISPOT assay + HLA restriction)	Tetramer staining	Intracellular cytokine staining
Controllers (<50–2,000 copies/ml)	34	400 (20–2,000)	684.5 (340–1,162)	30	11	6
Progressors (2,001– >50,000 copies/ml)	46	26,800 (2,100–4,540,000)	415.5 (225,934)	42	10	8
Total	80			72	21	14

[a]Patient demographics were as follows: by sex, 76.3% were female ($n = 61$) and 23.8% were male ($n = 19$), and by race, 97.5% were African and 1.2% each were Asian and Caucasian (percentages do not total 100% because of rounding). All study participants included in the tetramer and intracellular cytokine staining studies maintained viral control for a minimum of 1 year, including at the time point of analysis in the case of controllers, and had comparable CD4 cell counts ($P = 0.87$). Individuals utilized for the initial screening and HLA restriction assays were not necessarily the same individuals used to perform tetramer and intracellular cytokine staining.

for future HIV vaccine design. Currently, the development of assays that can fully quantify all the relevant epitope-specific responses remains a major challenge in natural infection and vaccine studies because of the tremendous complexity of CD4+ T cell biology. This study employed MHC class II tetrameric complexes to enumerate and characterize Gag p24-specific CD4+ T cell populations *ex vivo* in HIV-infected individuals bearing HLA DRB1*11:01, DRB1*13:01, and DRB1*03:01 alleles. Overall, our study shows that MHC class II tetramers are very sensitive for detecting directly *ex vivo* HIV-specific CD4+ T cells present at very low frequencies. Our approach provides an alternative method of identifying antigen-specific CD4+ T cells without relying on function and removes the bias associated with the *in vitro* stimulation required for functional assays and the limitation associated with the detection of only subsets of cells capable of secreting cytokines. A notable limitation of our study, however, is the lack of availability of a wide range of class II tetramers complexed with other MHC class II DRB1 alleles or the less well characterized DP and DQ alleles. Notwithstanding this limitation, the screening data from the ELISPOT assay show that we were able to measure most of the immunodominant responses which were restricted by DRB1 alleles pertinent to this African population for which we successfully generated a subset of relevant class II tetramers. Taken together, these data demonstrate that HIV-specific CD4+ T cell responses restricted to DRB1*11-Gag41 are associated with immune control of HIV-1 infection and highlight the advantages of class II tetramer technology in evaluating HIV-specific CD4+ T cell responses in future natural infection and vaccine studies.

MATERIALS AND METHODS

Cohort characteristics. A total of 80 subjects chronically infected with HIV-1 clade C (76.3% female [$n = 61$] and 23.8% male [$n = 19$] subjects; the percentages do not total 100% because of rounding) from the local Zulu/Xhosa population, recruited as part of the Sinikithemba cohort (35) in Durban, South Africa, were studied. The Biomedical Research Ethics (BREC) of the University of KwaZulu-Natal and the institutional review board (IRB) of Massachusetts General Hospital approved this study. All study participants were antiretroviral naive at the time of enrollment. Sociodemographic characteristics, plasma viral load measurements, and CD4+ T cell counts were obtained at the baseline. Follow-up CD4+ T cell counts and plasma viral load measurements were performed at 3 months and 6 months. The individuals recruited for this study were stratified into two major groups: 34 HIV controllers, defined here as individuals who maintained lower viral loads of <2,000 HIV RNA copies/ml for >1 year, and 46 HIV chronic progressors, defined here as individuals who maintained viral loads of >2,000 HIV RNA copies/ml, on the basis of the contemporaneous viral load (Table 3). HLA-DRB1 typing was performed for all participants as described previously (16). Cryopreserved peripheral blood mononuclear cell (PBMC) samples from each individual were utilized for all assays. An initial number of 72 participants were screened for HIV-specific CD4+ T cell responses using the ELISPOT assay. Individuals that made a CD4+ response were then screened using the HLA restriction assay to determine the restricting alleles and epitopes. For additional comparisons of controllers and progressors, data for an additional 8 individuals that were not utilized in the initial screening were added to the data set. Hence, the total number of participants in the study was 80 (Table 3). MHC class II tetramers were synthesized and tested for approximately one-quarter (21/80) of study subjects in this cohort with clade C infection who expressed the matching HLA class II DRB1 alleles.

CD8+ T cell depletion and modified IFN-γ ELISPOT assay. A modified IFN-γ ELISPOT assay was used to screen all chronically infected subjects. Cryopreserved PBMCs were thawed, and CD8+ T cells

were depleted using Miltenyi magnetically activated cell sorting CD8 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The initial screening was carried out using a megamatrix approach (36, 37) with consensus clade C HIV-1 peptides custom produced at the Massachusetts General Hospital Core facility. The megamatrix involved the use of 410 18-mer overlapping peptides (OLPs), which overlapped by 10 amino acids, that spanned the entire HIV-1 proteome. These were arranged into 72 pools of between 10 and 12 peptides in each pool, such that an individual peptide was uniquely represented in two different pools. Each megamatrix peptide pool was cocultured with 100,000 CD8-depleted cells per well in 96-well polyvinylidene plates (catalog number MAIP S45; Millipore, MA, USA) that had been precoated with 100 μl the MAb1-D1k anti-IFN-γ monoclonal antibody (MAb; 0.5 μg/ml; Mabtech, Stockholm, Sweden) overnight at 4°C. Positive responses from the initial megamatrix screening were confirmed using a separate ELISPOT assay at the single-peptide level. For screening at the single-peptide level, a total of 100,000 CD8-depleted PBMCs per well were plated in 96-well polyvinylidene plates (catalog number MAIP S45; Millipore, MA, USA) that had been precoated with 100 μl the MAb1-D1k anti-IFN-γ monoclonal antibody (0.5 μg/ml; Mabtech, Stockholm, Sweden) overnight at 4°C in the presence or absence of specific HIV-1-derived OLPs in a final volume of 200 μl R10 medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum [FCS], 2 mM L-glutamine, 50 U/ml of penicillin, 50 μg of streptomycin/ml, and 10 mM HEPES). Each well contained a single OLP (10 μl) at a final concentration of 2 μg/ml for each individual peptide. Cells in medium without antigenic stimuli represented negative controls. Phytohemagglutinin (PHA) antigen (Sigma-Aldrich, St. Louis, MO, USA), which served as a positive control for both cell viability and the functionality of the immunoassay, was added at a concentration of 5 μg/ml. The plates were incubated for 40 h at 37°C in 5% CO_2 to elicit the maximum amount of cytokine secretion. The ELISPOT assay plates were then processed as previously described (19). An antigen-specific $CD4^+$ T cell response was considered positive only if the level of the response was at least $\geq$3 times the mean background level and also if the standard deviation of the number of spot-forming cells (SFC) was $\geq$3 times the standard deviation of the number of SFC for the negative controls. The breadth of the responses was defined as the sum of the IFN-γ-positive peptide responses within a given individual across the entire HIV proteome or within a specified protein. Positive responses to two adjacent overlapping peptides were relatively rare in our cohort (2/30 responders), but where they occurred, the two overlapping peptides were treated as a single epitope to ensure that the breadth of $CD4^+$ T cell responses was not overestimated.

HLA genotyping. High-resolution (4-digit-allele) class I/II genotyping was carried out using standard sequence-based typing protocols (16). In brief, HLA class I genes were amplified by PCR with primers spanning exons 2 and 3, and HLA class II typing was based on exon 2. Sequencing results were interpreted using ASSIGN (version 3.5) software, developed by Conexio Genomics (Fremantle, Western Australia, Australia).

HIV-specific $CD4^+$ T cell lines and HLA-DR restriction assay. A modified HLA-DRB1 restriction assay was utilized as previously described (12) to define restricting HLA class II alleles. In brief, frozen CD8-depleted PBMC samples from subjects with known $CD4^+$ T cell responses were used to generate $CD4^+$ T cell lines. CD8-depleted PBMCs at a concentration of 2 million cells on a 24-well plate in R10 medium were stimulated with 10 μg/ml of peptide. The cells were incubated at 37°C in 5% CO_2. After 2 days, the cells were washed and fresh R10 medium supplemented with 100 U/ml recombinant IL-2 was added. The $CD4^+$ T cell lines were fed twice weekly with regular medium replenishment. After 14 days, the T cell lines were simultaneously assessed for their specificity and HLA-DR restriction using a large panel of L cell lines (LCLs), which are mouse fibroblasts, each stably transfected with a single recombinant human HLA-DR molecule, as previously described (21). Each LCL was pulsed with 10 μg/ml peptide for 90 min at 37°C in 5% CO_2 and washed five times to remove free peptide. Overlapping peptides from the HIV-1 clade C consensus sequence were used in the assay. Ten thousand peptide-pulsed LCLs were cocultured in triplicate with 50,000 cells of each respective $CD4^+$ T cell line per well on a precoated IFN-γ plate. As a negative control, each $CD4^+$ T cell line was cocultured in triplicate with the appropriate LCL in the absence of peptide. As a positive control, PHA was added at 2 μg/ml. The plates were incubated for 18 h at 37°C in 5% CO_2 and processed by the usual ELISPOT assay protocol described above. An Aid ELISPOT assay reader (Autoimmun Diagnostika, Germany) was used to determine the number of SFC per 50,000 cells of the $CD4^+$ T cell line. HLA-DR restriction was considered positive if the level was at least $\geq$3 times the mean background level and also $\geq$3 times the standard deviation for the negative-control wells.

MHC class II tetramers. The HIV clade C p24 Gag41 peptide (YVDRFFKTLRAEQATQDV) was complexed to recombinant human DRB1*11:01 or DRB1*13:01. In addition, the HIV clade C p24 Gag37 peptide (WIILG LNKIVRMYSPVSI) was complexed to recombinant human DRB1*13:01 and DRB1*03:01. These tetramers are referred to as the specific HLA together with the peptide designation here (e.g., DRB1*11:01-Gag41). All tetramers were conjugated to PE and APC at a concentration of either 454 nM or 432 nM. The tetramers were produced in the laboratory of Søren Buus under previously described conditions (38).

Tetramer and surface staining. Unless stated otherwise, PBMCs were incubated with MHC class II tetramers with a final staining concentration of 30 nM for 1 h at 37°C in medium. After the cells were washed with cold 2% FCS–phosphate-buffered saline (PBS) buffer, antibodies to cell surface molecules were added and the mixture was incubated at 4°C for 30 min. The following anti-human-conjugated antibodies were used: CD3-BV785-OKT3 (catalog no. 317330; BioLegend, San Diego, CA, USA), CD4-AF700-RPA-T4 (catalog no. 557922; BD, San Jose, CA, USA), and CD8-BV605-SK1 (catalog no. 564116; BD). PBMCs were also stained with LIVE/DEAD fixable aqua cell viability dye (catalog no. L34957; Invitrogen). Stained cells were washed with 2% FCS–PBS buffer and fixed with fixation medium A (catalog no. GAS001S-100; Invitrogen). Data were acquired on an LSR Fortessa flow cytometer (serial number

H647794E6049; BD). Flow data were analyzed using FlowJo software (version 10.1; TreeStar Ashland, OR, USA).

Intracellular cytokine staining. Briefly, PBMCs were incubated overnight with a clade C consensus Gag peptide pool or Gag41 as the specific antigen/stimulus and staphylococcal enterotoxin B (SEB) (Sigma) as a positive control in the presence of a Golgi apparatus stop protein transport inhibitor (BD Biosciences) and Golgi apparatus plug protein transport inhibitor (BD Biosciences), according to the manufacturer's instructions. Anti-CD107a-PE-Cy5-H4A3 (catalog no. 555802; BD) was also added at the beginning of the stimulation period. After the stimulation, intracellular cytokine staining was performed according to the BD Biosciences ICS protocol. Unstimulated cells were used as a control. Cells were stained with LIVE/DEAD fixable aqua dead cell viability dye (catalog no. L34957; Invitrogen) and fluorescent antibodies against CD3-BV711-OKT3 (catalog no. 317328; BioLegend), CD4-BV650-SK3 (catalog no. 563875; BD), and CD8-AF700-RPA-T8 (catalog no. 557945; BD). Cells were permeabilized and fixed with BD intracellular staining reagents according to the manufacturer's instructions (BD Biosciences) and stained with cytokine-specific antibodies against IFN-γ–PE–Cy7–B27 (catalog no. 557643; BD), IL-21–PE–3A3-N2.1 (catalog no. 560463; BD), and IL-2–fluorescein isothiocyanate–5344.111 (catalog no. 340448; BD). Cells were acquired on an LSR Fortessa flow cytometer (serial number H647794E6049; BD). Flow data were analyzed using FlowJo software (version 10.1; TreeStar).

***Ex vivo* expansion of HIV-specific CD4 T cells.** MHC class II tetramer staining was performed prior to culture. Cells were then cultured at 37°C in 5% CO_2 for 14 days in the presence of HIV clade C p24 Gag37 and 41 OLPs, as described above for the HIV-specific CD4+ T cell lines. On day 14, the cells were washed three times with fresh R10 medium and rested at 37°C in 5% CO_2 overnight in fresh R10 medium. On the following day, MHC class II tetramer staining was performed to determine the levels of expansion. The cells were then stimulated for 6 h with the same peptides used for expansion, and the cytolytic activity of the expanded cells was assessed by ICS staining for granzyme A-AF488-CB9 (catalog no. 507212; BioLegend), granzyme B-AF700-GB11 (catalog no. 560213; BD), and CD107a-PE-Cy5-H4A3 (catalog no. 555802; BD).

Statistical analysis. Statistical analysis and graphical presentation were performed using GraphPad Prism (version 5.0) software (GraphPad Software Inc., La Jolla, CA, USA). Spearman's rank correlation was used to assess the relationship between the immune responses and the viral load. Statistical analysis of significance was calculated using the Kruskal-Wallis test with Dunn's *post hoc* analyses for multiple comparisons. The significance of the nonrandom associations (contingency) between controllers and progressors was computed using Fisher's exact test. In addition, the Mann-Whitney U test was utilized to compare differences between two groups. Statistical significance was set at a P value of <0.05.

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